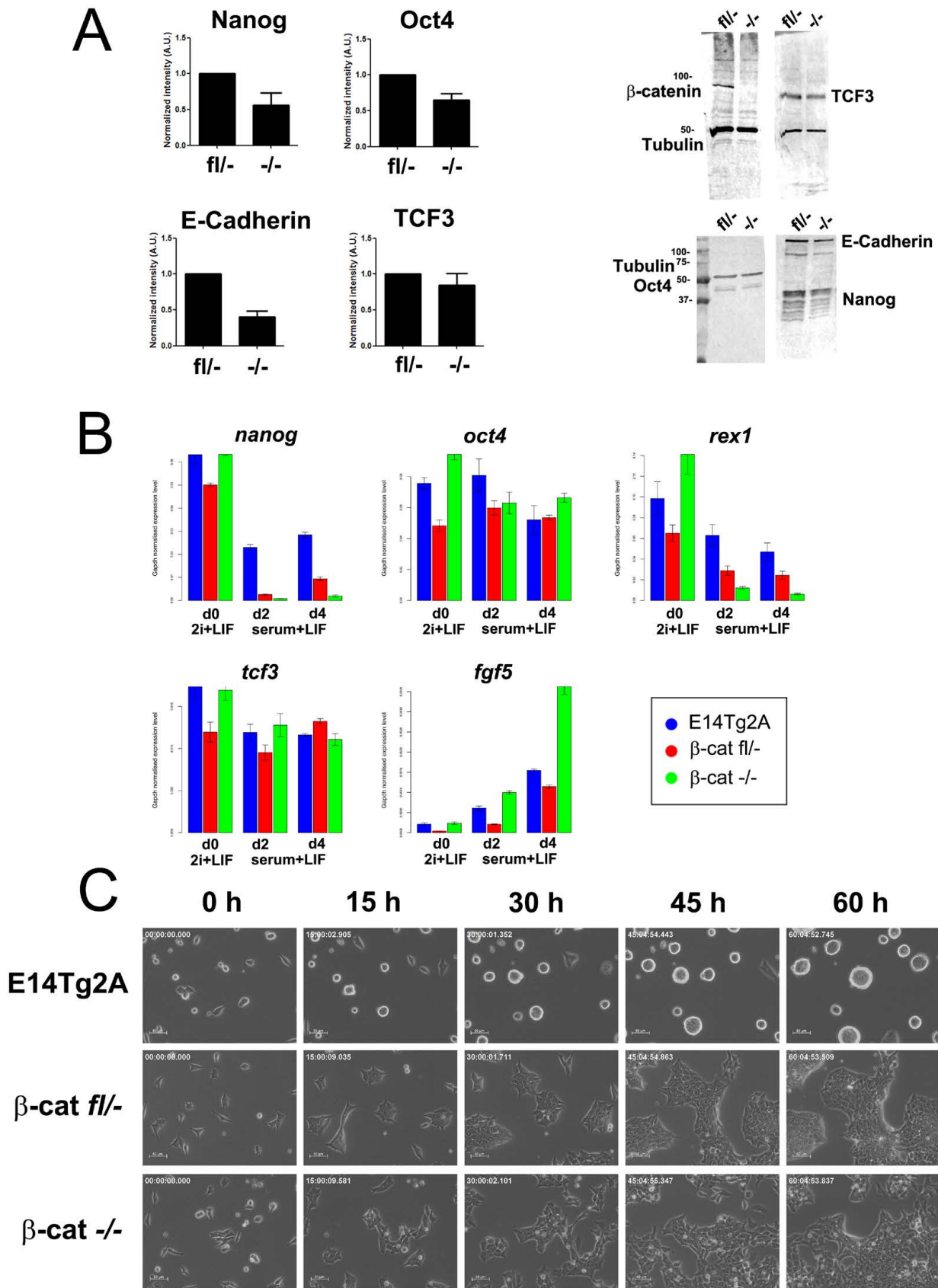


Fig. S1. Characterization of the effects of Wnt signalling inhibitors on mESC pluripotency and differentiation. (A) TNGA cells were grown in N2B27 supplemented with either Wnt3A-conditioned medium or recombinant Wnt3A for 2 days. (B) Profiles of TNGA (Nanog::GFP) cells comparing differentiation in N2B27 under the different conditions as indicated after 2 days in culture. The effect of IWP2 (an inhibitor of the Wnt chaperone porcupine) is dependent on the culture medium. Although there is little effect in LIF+BMP, there is a slight loss of pluripotency in serum+LIF. Over longer periods of time, IWP2 leads to a loss of pluripotency (not shown) in agreement with published results. We believe this is not due to the loss of pluripotency but to an increased flow of LN cells into differentiation that, slowly, drains the pool of pluripotent cells. (C) 10,000 cells/well (96-well plate) were plated on day 0. In parallel, 10,000 cells were transfected for the 14x TOP-Luciferase and SV40-Renilla control vectors. On day 1, media were replaced with 50 μ g of C3 and control DMSO in serum+LIF media. Cells were processed on day 2 for immunostaining and luciferase assays.



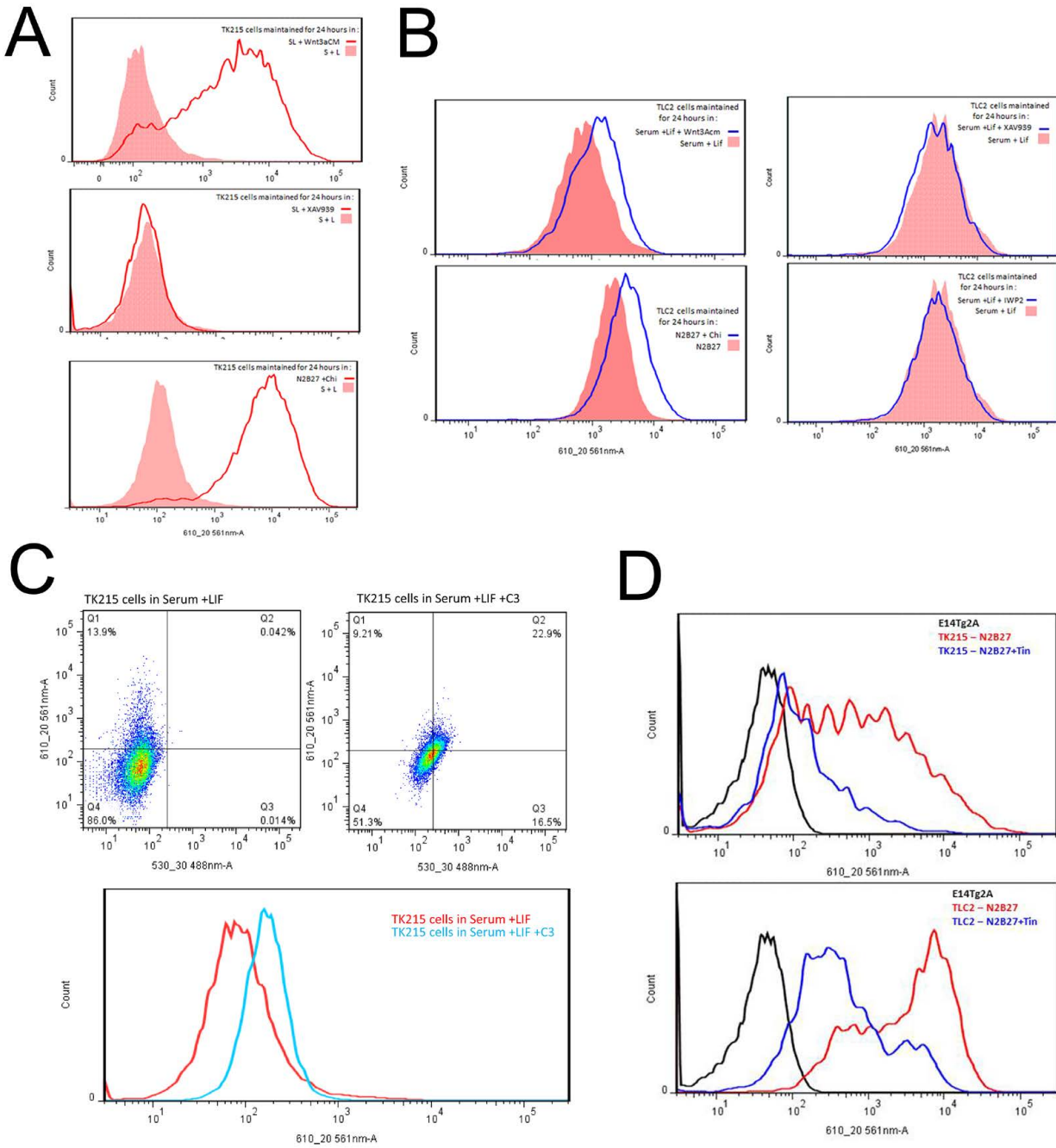


Fig. S3. Characterization of fluorescent Wnt reporter mESC lines. (A,B) Characterization of Wnt reporter lines in TK215 (A) and TLC2 (B) under conditions that promote or inhibit Wnt activity. (C) The addition of ICRT3 to the cells for 3 days causes an increase in the autofluorescence of the cells. Over shorter time periods, this increase is not apparent (see Fig. 1E).

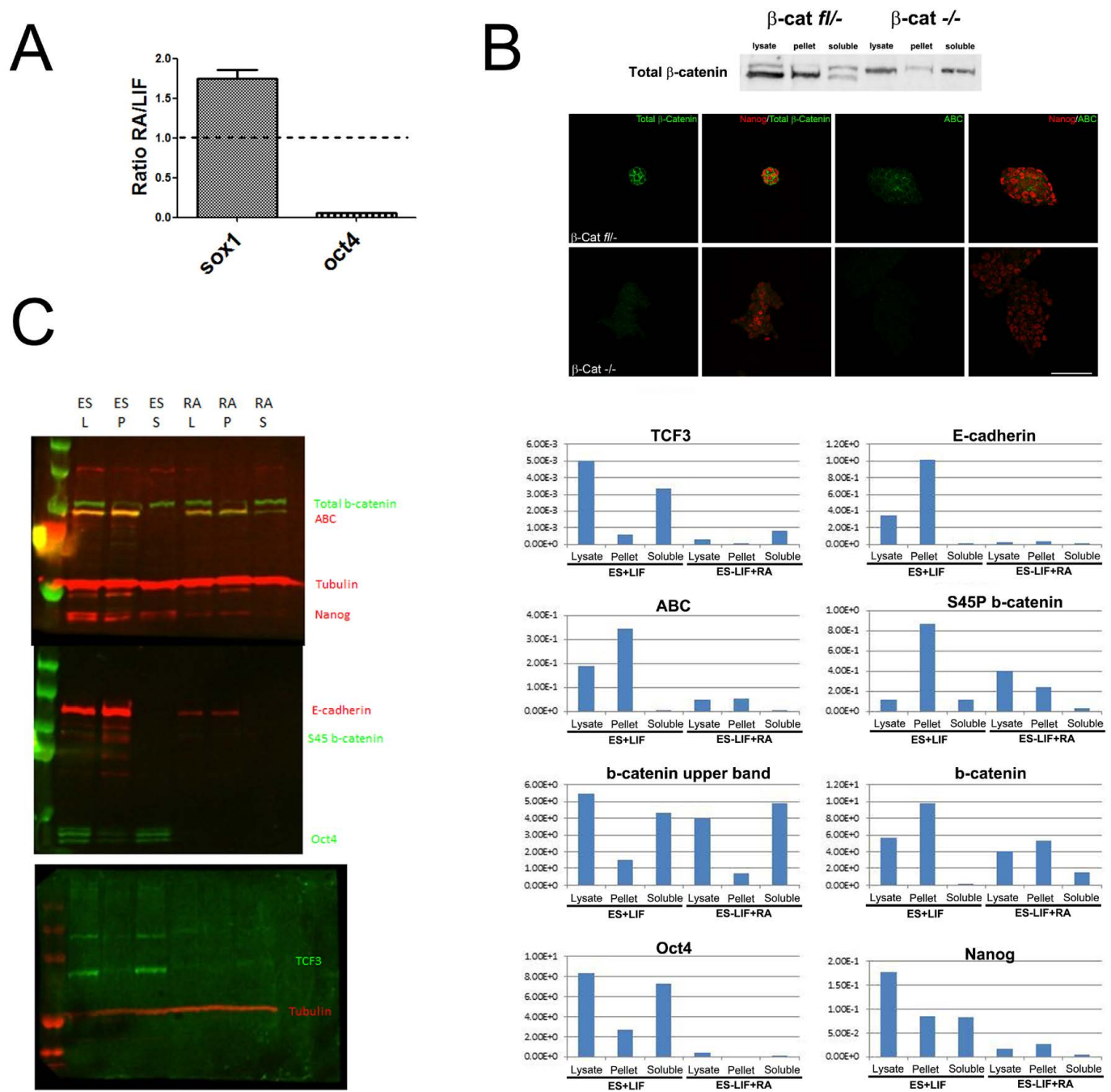


Fig. S4. Characterization of Serum+RA induced differentiation. (A) E14Tg2A cells were grown in self-renewing (serum+LIF) or differentiating conditions (serum+1 mM RA) for 3 days. Total RNA was isolated and qPCR was performed using specific primers for *Sox1* and *Oct4*. Normalization to *Gapdh* was performed and the ratio between serum+1 mM RA and serum+LIF was plotted. (B) Western blots of β -catenin^{fl/-} and β -catenin^{-/-} cells with a Total β -catenin antibody (Sigma cc2206). The upper band has a non-specific component (see main text). β -Catenin^{fl/-} and β -catenin^{-/-} cells were stained for total β -catenin (green channel) and Nanog (red channel), and for ABC (green channel) and Nanog (red channel) to show the specificity of these antibodies. This non-specific component of the upper band is also not recognized by total β -catenin (Abcam 19450, data not shown). For a further discussion of the specificity of β -catenin antibodies see Maher et al. (Maher et al., 2009a) (C) Gel images (left) and quantifications (right) of the experiment shown in Fig. 4A. Western blot membranes were scanned in the Odyssey System. The intensity of each band was normalized to tubulin. ES, serum+LIF; RA, serum+1 mM RA.

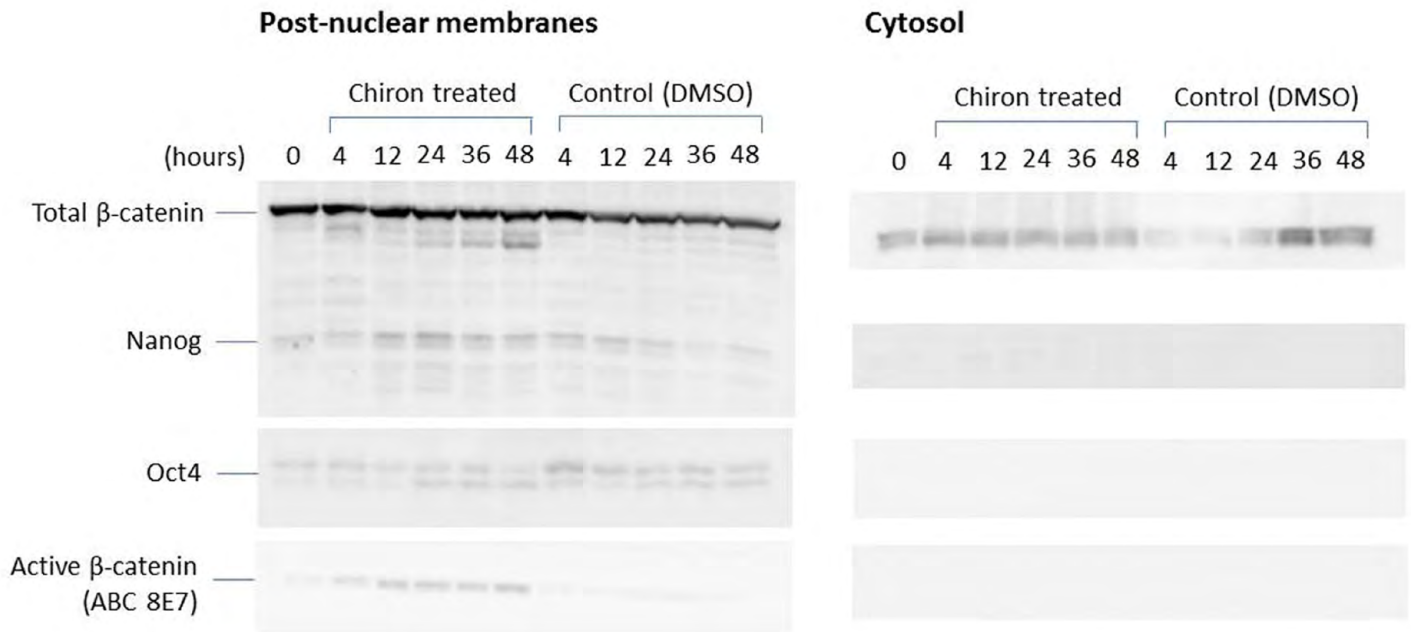


Fig. S5. Nanog and Oct4 are associated with membranes in mESCs. Post-nuclear membranes and cytosolic proteins fractionated by differential centrifugation as previously described (for details, see Berrios, 1997). Post-nuclear membranes samples were analyzed by mass spectrometry as previously described (for details, see Hall et al., 2009) to confirm they were free of nuclear contamination and strongly enriched in organellar proteins, including plasma membrane residents. Concentration of proteins was determined by BioRad DC assay and 2 μ g protein was used for western blot analysis. Post-nuclear membranes were confirmed to be free of nuclear contamination and strongly enriched in organellar proteins, including plasma membrane residents by mass spectrometry analysis. Here, Oct4 and active β -catenin were found to localize to post-nuclear membranes but not the cytosol, whereas Nanog was readily detectable in post-nuclear membranes but almost undetectable in the cytosolic fraction.

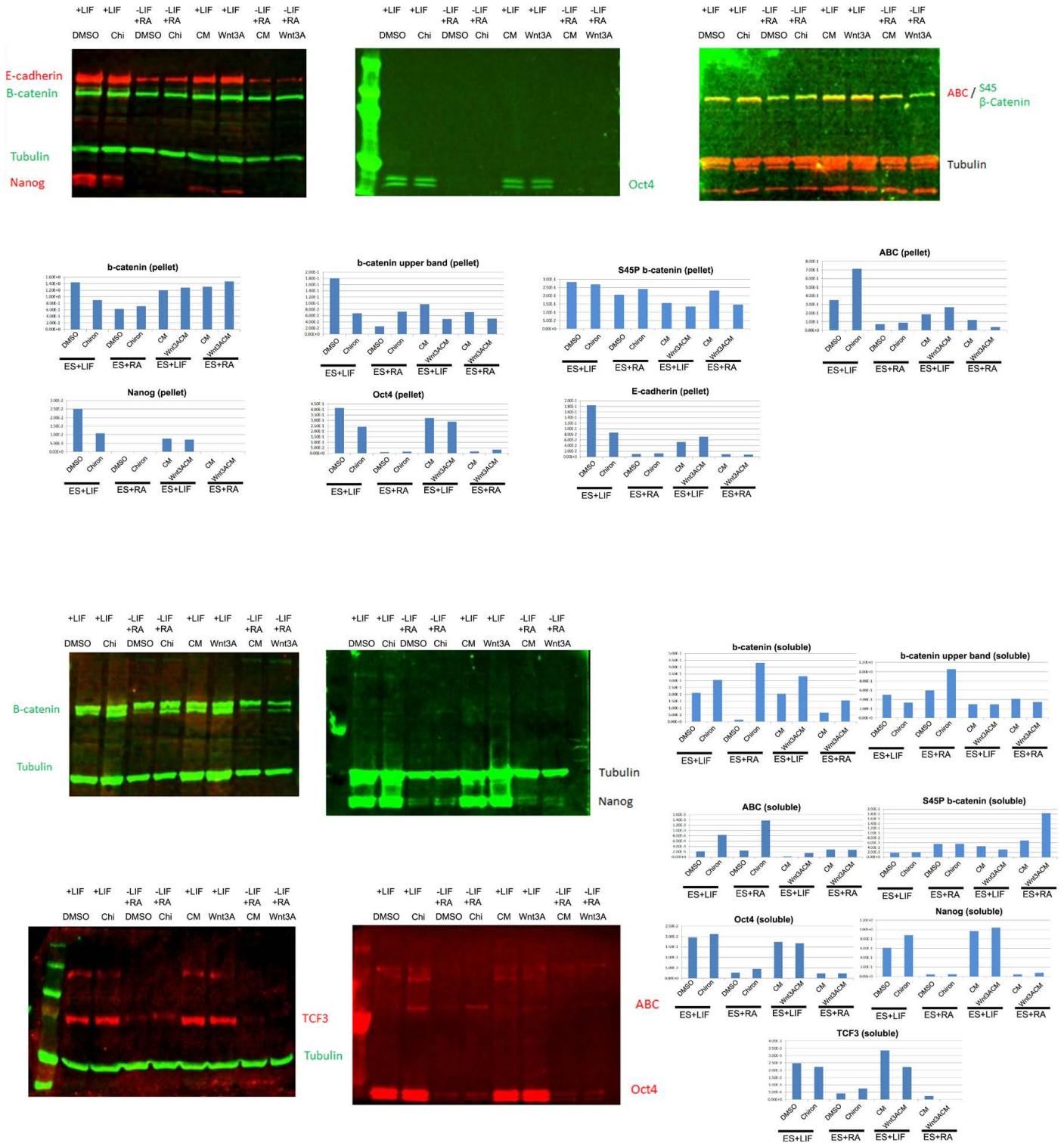


Fig. S6. Quantifications of western blots from Fig. 4C. Gel images and quantifications of western blots shown in Fig. 4C for membrane-associated proteins (pellet, upper panels) and soluble fractions (lower panels). Western blots membranes were scanned in the Odyssey System. Intensity of each band was normalized to tubulin. Primary antibodies used are: anti-Nanog (eBiosciences 14-5761, 1:100), anti-Oct4 (Santa Cruz 5279, 1:100); anti-SSEA1 (Santa Cruz 21702, 1:100); anti-active β -catenin (ABC) recognizing dephosphorylated residues in the interval 36-44 (Millipore 05-665, 1:300) (van Noort et al., 2007; Maher et al., 2009; Maher et al., 2010); anti-PSer45- β -catenin, recognizes PThr41 and PSer45 (Cell Signaling 9565, 1:200) (Maher et al., 2009; Maher et al., 2010); anti-total- β -catenin, recognizing epitopes in the C terminus of β -catenin residues 768-781 (Sigma C2206, 1:1000); anti-TCF3 (Santa Cruz sc-8635, 1:40 from the preabsorbed antibodies in *Tcf3*^{-/-} cells). All antibodies, except anti-Oct4, were tested on mES cells mutant for the relevant protein to verify specificity. Secondary antibodies labeled with IRDye infrared dyes were used and membranes were scanned using the Odyssey Imaging System (LI-COR Biosciences). Quantification was performed using the LI-COR software normalizing with respect to tubulin intensity.

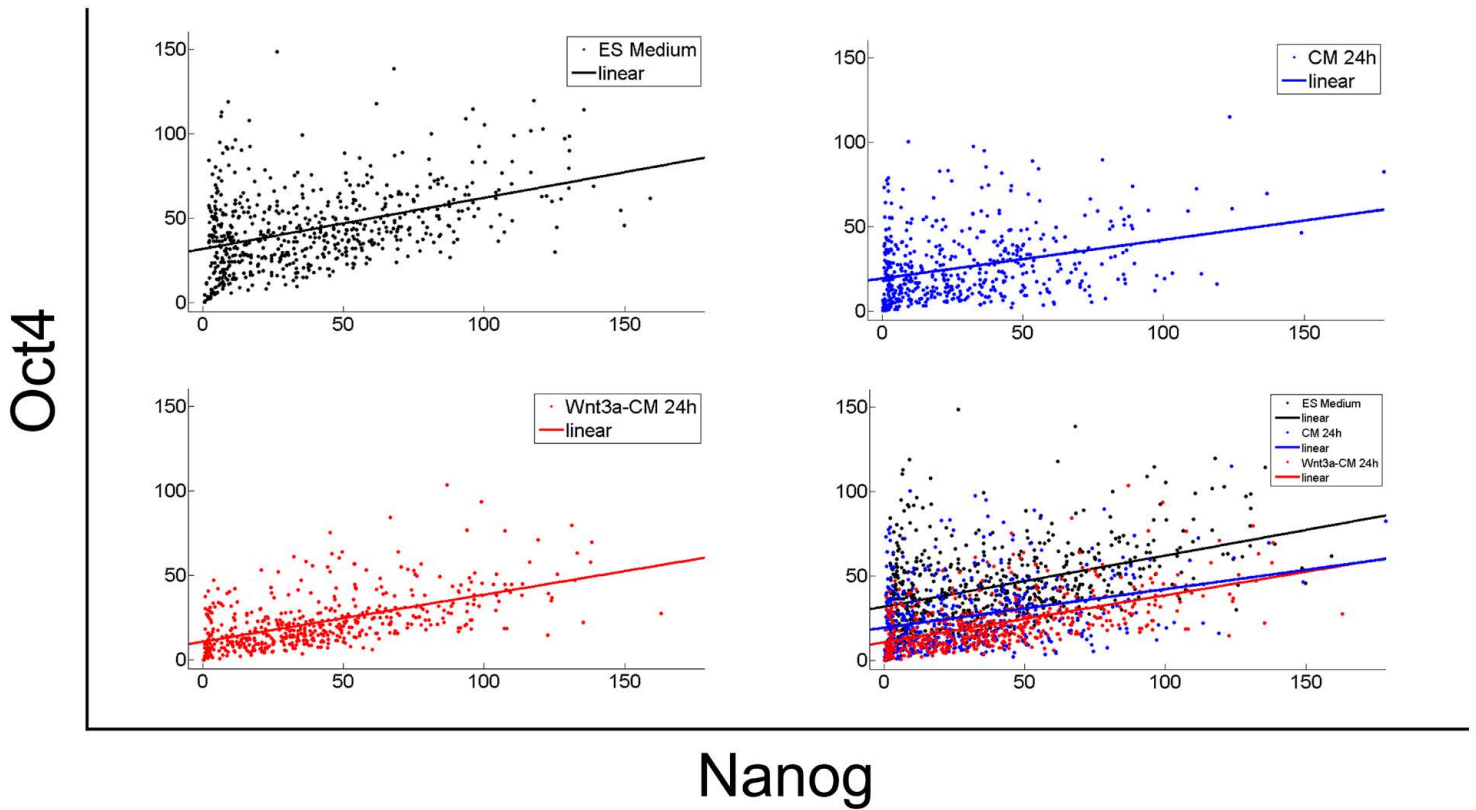


Fig. S7. The correlation between Nanog and Oct4 protein levels in single cells increases with Wnt signalling. Activation of Wnt signaling by treating E14Tg2A cells with Wnt3A-conditioned medium (Wnt, red dots) for 24 hours increases the correlation between Oct4 and Nanog (R^2 : 0.2931). This effect is not mimicked by the control conditioned medium alone (CM, blue dots; R^2 , 0.1033; P value=0.0001, Fisher r-to-z transformation). This result shows that the increased in correlation between Oct4 and Nanog is due to the activation of Wnt signaling. The black dots indicate cells grown in serum+LIF.

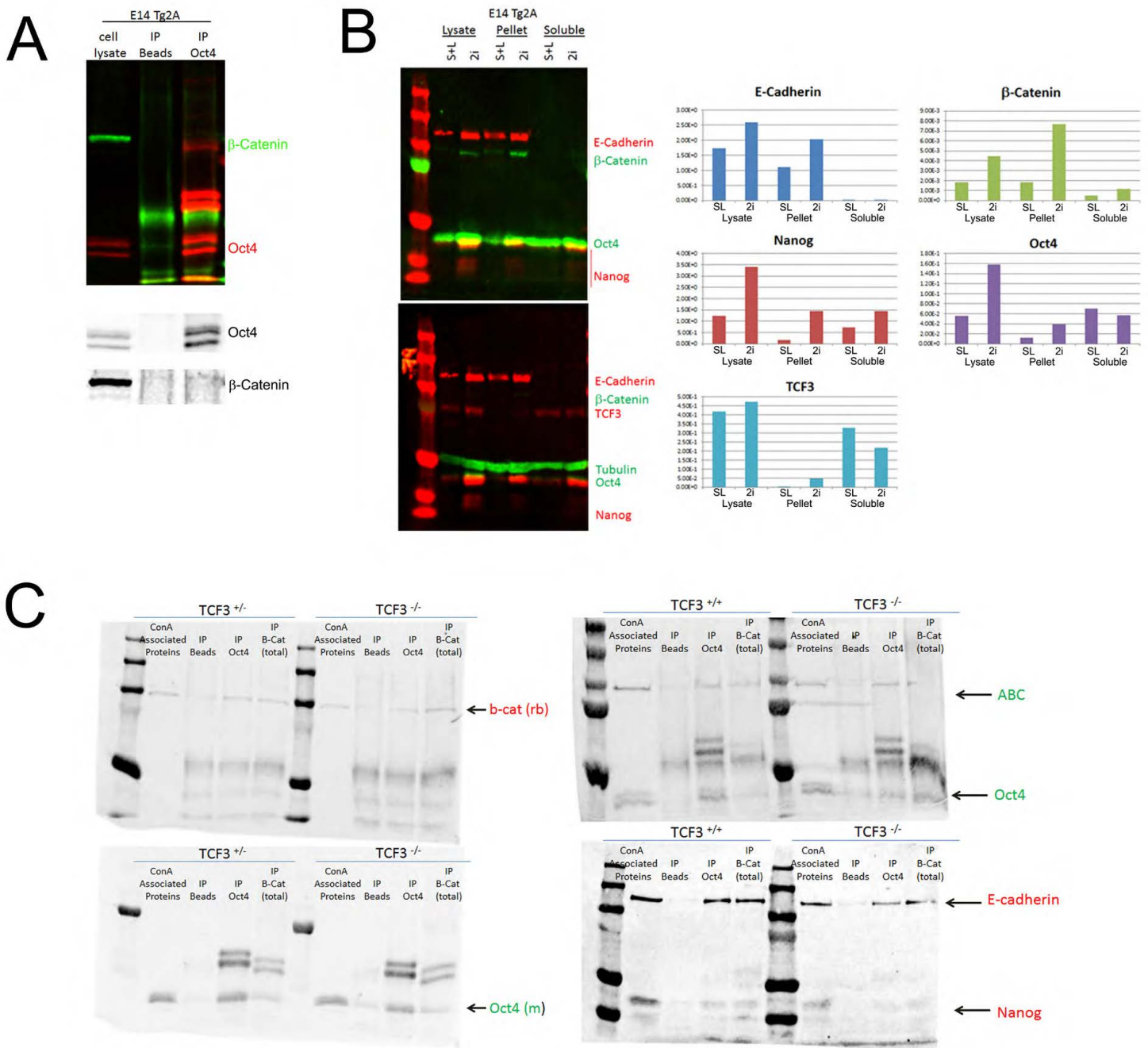


Fig. S8. Quantification of western blots from Fig. 6. (A) Immunoprecipitation of Oct4-containing protein complexes from total cell lysates of E14Tg2A cells grown in serum+LIF. There is a faint band of β -catenin in the Oct4 IP. (B) Uncropped blots from Fig. 6A. Protein expression was quantified and normalized to tubulin expression (see graphs alongside blots). These samples were obtained as follows. Immunoprecipitations were carried out on either whole-cell lysates or ConA-bound membrane-associated proteins. Anti-Oct4 (Santa Cruz sc5279) and protein A/G magnetic beads were incubated with the input for 1 hour at room temperature, the magnetic beads were then separated using a magnet against gravity (to prevent unbound ConA-sepharose beads contaminating the immunoprecipitate) and washed three times in PBS. Immunoprecipitated proteins were released in $2\times$ Laemmli's buffer and identified by western blot (C) Uncropped blots from Fig. 6B.

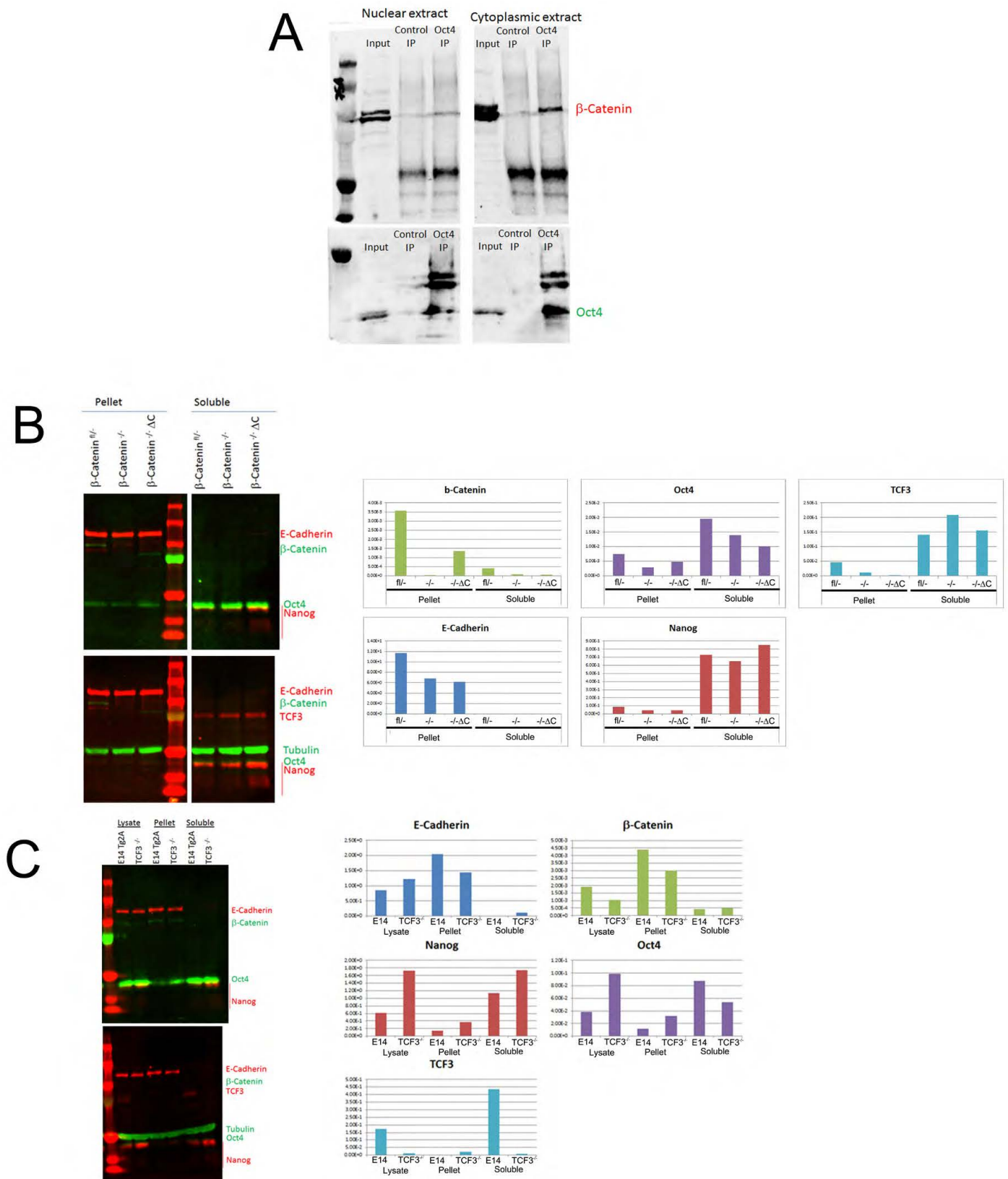


Fig. S9. A β -catenin and Oct4 complex is found in the cytoplasmic fraction of mESCs. (A) E14Tg2A cells maintained in 2i were fractionated using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). Immunoprecipitation of Oct4 protein was then carried out using anti-Oct4 (Santa Cruz sc5279) and protein A/G magnetic beads. These were incubated with the input for 1 hour at room temperature, the magnetic beads were then separated using a magnet and washed three times in PBS. Immunoprecipitated proteins were released in $2\times$ Laemmli's buffer and identified by western blot. A cytoplasmic Oct4/ β -catenin complex is present in the Oct4 immunoprecipitate. E-cadherin and Nanog were not found to be associated with this complex under these conditions. Detection of E-cadherin in cytoplasmic, but not nuclear, fractions, and mSin3A in nuclear, but not cytoplasmic, extract confirmed effective nuclear/cytoplasmic fractionation (data not shown). (B,C) Uncropped blots from Fig. 6C,D. Protein expression was quantified and normalized to tubulin expression (see graphs alongside blots).

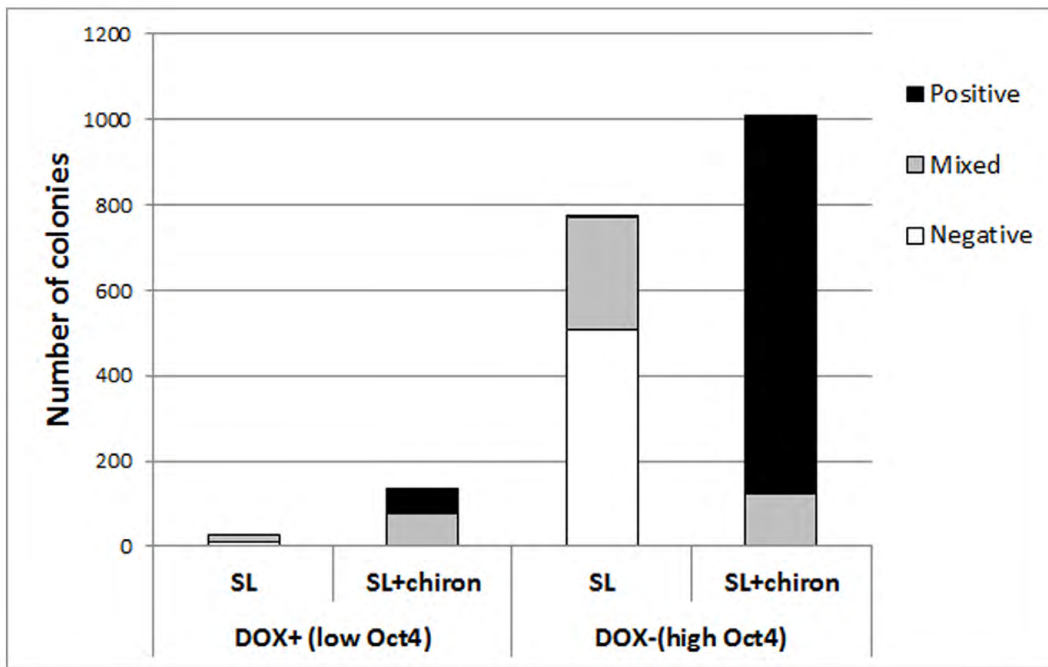
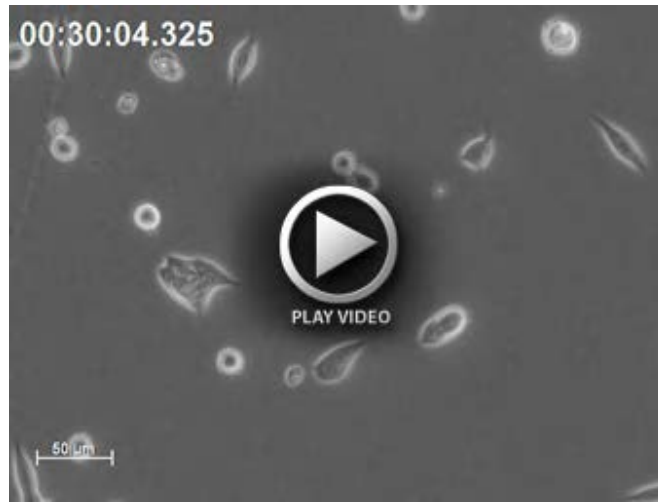


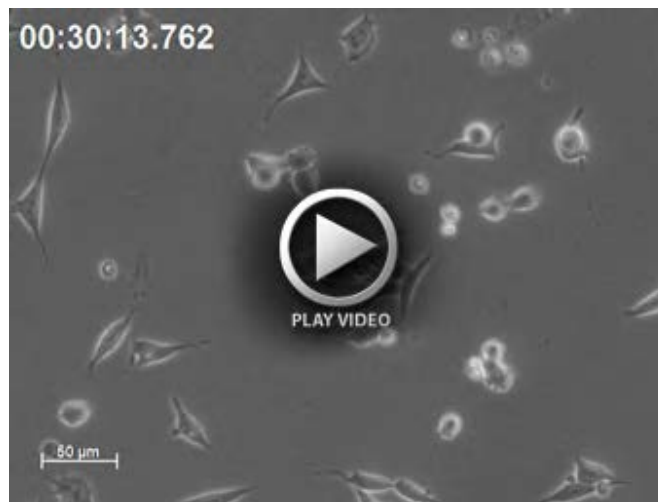
Fig. S10. Oct4 induced differentiation is inhibited by increasing β -catenin levels. AG23191 cells overexpressing Oct4 from a TetOFF system were grown in serum+LIF in the presence of absence of chiron. Colonies were assayed for alkaline phosphatase and the quantification of the assay in Fig. 7D is shown.



Movie 1



Movie 2



Movie 3

Movies 1-3. Reduction or loss of b-catenin results in enhanced differentiation even in conditions that promote pluripotency. E14Tg2A (Movie 1), β -catenin^{fl/-} (Movie 2) and β -catenin^{-/-} (Movie 3) cells were grown in 2i+LIF on fibronectin and filmed during 3 days. Note in wild-type mESC (E14Tg2A) the cells grow in tight ball like colonies, with reduction or loss of β -catenin the cells grow in loose colonies and display a differentiated morphology.

Table S1. Primers used

Gene	Sequence
<i>Nanog</i>	TACCTCAGCCTCCAGCAGAT GCAATGGATGCTGGGATACT
<i>Oct4</i>	CCAATCAGCTTGGGCTAGAG CTGGGAAAGGTGTCCTGTA
<i>Sox2</i>	CATGAGAGCAAGTACTGGCAAG CCAACGATATCAACCTGCATGG
<i>Rex1</i>	GCGGTGTGTACTGTGGTGTC GACAAGCATGTGCTTCCTCA
<i>Fgf5</i>	GCTCAATGATCAGAAGGAGGA TCAGCTGGTCTTGAATGAGG
<i>Axin2</i>	ACACATGCAGAAATGGGTCA ACGTACGGTGTAGCCTTTGG
β -catenin	GGTGGGCTGGTATCTCAGAA CTTGATCCATTCGTGTGC
<i>Tcf3</i>	ACGGTTCTGGATGAGACAGG AAGCAGGGAGCTGTTCAGTG
<i>Gapdh</i>	AACTTTGGCATTGTGGAAGG GGATGCAGGGATGATGTTCT